PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent Application of Simon Rhodes, et al.	: Group Art Unit: Not Yet Assigned
Appln. No.:	Not Yet Assigned	: Examiner: Not Yet Assigned
Filed:	Herewith	:
For:	GENERATION OF DIAGNOSTIC TOOLS TO ASSAY THE HUMAN LHX3/P-LIM/LIM-3 FACTOR	: Attorney Docket : No. 53884-5003

PRELIMINARY AMENDMENT

Preliminary to the examination of the above-referenced application, please amend the application as follows:

In the Specification:

Please amend the Brief Description of the Drawings section as indicated on the "marked-up" copy of the Brief Description of the Drawings. Added text is underlined and deleted text is ~~struck through~~. A clean copy of the amended section is also included herewith.

REMARKS

By way of this Preliminary Amendment, Applicants provide a substitute Brief Description of the Drawings section. Marked up and replacement pages containing this section are enclosed herewith.

The amendments made to the Brief Description of the Drawings serve to correlate the amendments made to formalize the drawings with the specification. The amendments reflect re-numbering of the Figures, deleting reference to color, and movement of text from the figures to the specification. Therefore, Applicants submit that no new matter has been added by way of this Amendment.

Favorable examination of the claims on the merits is respectfully requested.

Respectfully submitted,

SIMON RHODES, ET AL.

August 17, 2001
Date

By:

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Enclosures: Marked up copy of the Brief Description of the Drawings
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BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiment(s) which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown.

In the drawings:

Figure 1 is a diagram depicting the predicted amino acid sequence of porcine Lhx3 (SEQ ID NO:2) and comparison to murine P-Lim/Lhx3/LIM-3 (now referred to herein as mLhx3a) as described in Bach et al. (1995, Proc. Natl. Acad. Sci. USA 92:2720-2724) (M, GenBank Accession Number 780314) (SEQ ID NO:3), chicken LIM-3 (C, GenBank Acc. No. 1708828 (SEQ ID NO:4)), *Xenopus* lim-3 (X, GenBank Acc. No. 547856 (SEQ ID NO:5)) and zebrafish LIM-3 (Z, GenBank Acc. No. 2497671 (SEQ ID NO:6)). Alignment is based on that of Glasgow et al. (1997, Dev. Biol. 192:405-419) for zebrafish LIM-3. The LIM domains and homeodomain are bracketed. Dashes indicate identity and asterisks denote gaps introduced to optimize alignment. The GenBank accession number for the porcine Lhx3 nucleotide sequence is AF063245.

Figure 2 is an image depicting a Northern analysis of Lhx3 messenger RNA in porcine pituitary. RNA obtained from the indicated tissues was separated on denaturing gels, transferred to nylon membranes and probed sequentially using radiolabeled cDNA probes. A blot containing 30 μg of RNA per lane was used for the Lhx3 and prolactin probes; the Pit-1 and growth hormone blot contained 15 μg per lane. The migration positions of ribosomal RNAs are indicated on the left side of the image. An image of an ethidium bromide stained gel depicting ribosomal RNAs for the Lhx3/prolactin lanes used for Northern blotting is depicted at the bottom of the figure to demonstrate lane loading.

Figure 3A, comprising Figures 3Ai and 3Aii, is a diagram depicting activation of alpha-glycoprotein subunit (αGSU) gene promoter luciferase plasmids, the structure of which is depicted at the top of the figure, by pLhx3. Briefly, 293 cells were transiently transfected with porcine αGSU reporter gene construct and the indicated vectors (*i.e.*, control vector without insert, pLhx3 vector, and ΔLIM pLhx3 vector).

Promoter activity was assessed by measurement of luciferase activity after 48 hours. Luciferase activities disclosed are mean light units/10 seconds/μg total protein of triplicate assays ± S.E.M. Representative experiments of at least nine experiments are depicted.

Figure 3B, comprising Figures 3Bi and 3Bii, is a diagram depicting activation of alpha-glycoprotein subunit (αGSU) gene promoter luciferase plasmids, the structure of which is depicted at the top of the figure, by pLhx3. Briefly, 293 cells were transiently transfected with mouse αGSU reporter gene construct and the indicated vectors (*i.e.*, control vector without insert, pLhx3 vector, and ΔLIM pLhx3 vector). Alpha-GSU promoter activity was assayed by measurement of luciferase activity after 48 hours. Luciferase activities are mean light units/10 seconds/μg total protein of triplicate assays ± S.E.M. Representative experiments of at least twelve experiments are depicted.

Figure 3C is an image of a Western blot depicting expression of pLhx3 protein of predicted molecular weight in 293 cells transfected with myc-epitope-tagged pLhx3 expression vectors but not in cells transfected with control vector. The protein was detected using an anti-myc monoclonal antibody.

Figure 4A is an image depicting expression of recombinant porcine pLhx3 proteins using SDS-polyacrylamide gel analysis of GST fusion proteins containing either pLhx3 (GST-pLhx3) or ΔLIM pLhx3 (GST-ΔLIM). An image of a Coomassie blue stain of a protein gel is depicted. The migration of molecular weight standards is indicated (*i.e.*, 69 kDa and 46 kDa).

Figure 4B is an image depicting an analysis of recombinant porcine pLhx3 protein binding to DNA target sequences. The image depicts electrophoretic mobility shift assay using labeled wild type and mutant oligonucleotides representing the –351 to -324 bp region of the porcine αGSU gene promoter. Probes were incubated with the indicated proteins and competitor DNA and the resulting complexes were separated from free probe (F) by electrophoresis. The panel on the right of the figure was exposed three times as long as the left-hand panel to detect weak binding of GST-pLhx3 to the porcine αGSU binding site (which is indicated by an arrow).

Figure 5, comprising Figures 5A and 5B, is a diagram depicting synergistic activation of a *prolactin* enhancer/promoter reporter gene by Pit-1 and pLhx3

or ΔLIM pLhx3. Briefly, 293 cells were transiently transfected with a rat *prolactin* enhancer/promoter reporter gene plasmid depicted at the top of the figure, and the indicated expression vectors. Promoter activity was assayed by measurement of luciferase activity after 48 hours. Activities are mean light units/10 seconds/μg total protein of triplicate assays ± S.E.M. A representative experiment of at least six experiments is depicted.

Figure 6, comprising Figures 6A, 6B, and 6C, is a series of images depicting *in vitro* binding assays demonstrating interaction of pLhx3 with NLI and Pit-1 proteins. Radiolabeled proteins were generated by translation in the presence of [^{35}S]-methionine. The radiolabeled protein was incubated with the indicated GST fusion proteins or with excess GST alone as a control. After washing, bound proteins were separated by electrophoresis and were visualized by fluorography. The migration positions of molecular weight standards (in kDa) are depicted at the right of each figure.

Figure 7, comprising Figures 7A and 7B, is a diagram depicting the nucleic acid sequence of porcine Lhx3 (SEQ ID NO:2).

Figure 8 is a diagram depicting the amino acid sequence of porcine Lhx3 (SEQ ID NO:1).

Figure 9A is a schematic depiction of the domain structures of hLhx3a and hLhx3b. The hatched regions represent domains unique to each form: L, LIM domain; HD, homeodomain; LSD, Lhx3/LIM3-specific domain.

Figure 9B is a diagram depicting the amino acid sequences and alignment of the sequences of mammalian Lhx3 proteins. Comparison of human Lhx3a/b (Ha (SEQ ID NO: 10), Hb (SEQ ID NO: 12)), murine Lhx3a and b as described by Zhadanov et al. (1995, Dev. Dynamics 202:354-364) (Ma, GenBank Acc. No. L38249 (SEQ ID NO:3); Mb, GenBank Acc. No. L38248 (SEQ ID NO:21)), and porcine Lhx3 (P, AF063245 (SEQ ID NO:1)). The LIM domains (L), homeodomain (HD), and Lhx3/LIM3-specific domain (LSD) are bracketed. Dots indicate identity; dashes denote gaps introduced to optimize alignment.

Figure 9C is a diagram depicting the similarity of Lhx3/LIM3 family proteins. The numbers indicated above each domain indicate the percentage identity to hLhx3 for that domain. The organisms are abbreviated as follows with the GenBank

Accession Numbers provided in parentheses: c, Chicken (GenBank accession number 1708828 (SEQ ID NO:4)); X, *Xenopus laevis* (GenBank Accession No. 547856 (SEQ ID NO:5)); z, Zebrafish (GenBank Accession No. 2497671 (SEQ ID NO:6)); mLhx4, mouse Lhx4 (GenBank Accession No. AF 135415 (SEQ ID NO:20)); d, *Drosophila melanogaster* (GenBank Accession No. AF 109306 (SEQ ID NO:21)).

Figure 10, comprising Figures 10A, 10B, and 10C, is a diagram depicting the nucleic acid sequence of human Lhx3a (SEQ ID NO:9) cDNA. The start nucleotide is located at 1 nts and is designated at the commencement of upper case. The LIM Domain 1 located at nucleotides 91-223 is underlined as is LIM Domain 2 at nucleotides 268-432. The homeodomain at nucleotides 466-660 is also underlined. The stop nucleotide is located at position 1194.

Figure 11, comprising Figures 11A, 11B, and 11C, is a diagram depicting the nucleic acid sequence of human Lhx3b (SEQ ID NO:11). The start nucleotide at position 1 is designated by upper case letters. The start nucleotide is located at 1 nts and is designated at the commencement of upper case. The LIM Domain 1 located at nucleotides 106-248 is underlined as is LIM Domain 2 at nucleotides 283-447. The homeodomain at nucleotides 481-675 is also underlined. The stop nucleotide is located at position 1209.

Figure 12A is an image of a Southern blot depicting the fact that hLhx3 is encoded by single gene. Human genomic DNA was digested with EcoRI and blotted to a nylon membrane. The blot was probed with a hLhx3 cDNA probe at high stringency. The migration position of molecular weight standards (kb) is given.

Figure 12B is an image of a Northern blot depicting analysis of hLhx3 gene expression. Human kidney (lane 2) or pituitary (Pit.; lane 1) total (20 μg) or poly A^+ RNA (1 μg; lanes 3 and 4) was separated on a denaturing gel, transferred to a nylon membrane, and probed with the indicated radiolabeled cDNA probes (*e.g.*, Lhx3 and Pit-1). The migration positions of ribosomal RNAs (*i.e.*, 28S and 18S) are indicated. The arrowhead indicates hLhx3 RNA.

Figure 12C is an image of SDS-PAGE analysis of human Lhx3a and hLhx3b proteins. [^{35}S]-methionine radiolabeled hLhx3a and hLhx3b isoforms (lanes 1 and 2) and myc epitope-tagged derivatives (a-myc, b-myc; lanes 4 and 5) were generated

by *in vitro* transcription/translation. The proteins were separated by SDS electrophoresis, and dried gels were visualized using fluorography. The migration positions of protein standards (in kilodaltons) are shown. Lane 3 is control.

Figure 13A is an image depicting the localization of control green fluorescent protein (GFP) in cells. Human 293T cells were transiently transfected with expression vectors encoding multimerized green fluorescent protein (GFP) as a control (4xGFP). The cells were examined using phase contrast microscopy. Bar = 10 μm.

Figure 13B is an image depicting the localization of control GFP protein using fluorescence microscopy. Human 293T cells were transiently transfected with expression vectors encoding multimerized green fluorescent protein (GFP) as a control (4xGFP). The fluorescence was visualized using krypton-argon laser scanning confocal microscopy. 4xGFP control is restricted to the cytoplasm. Bar = 10 μm.

Figure 13C is an image depicting the localization of hLhx3a-GFP in cells. Human 293T cells were transiently transfected with expression vectors encoding hLhx3a-4xGFP. The cells were examined using phase contrast microscopy to visualize cell structures. Unlike control 4xGFP, which is restricted to the cytoplasm, hLhx3a-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 13D is an image depicting the localization of hLhx3a-GFP using fluorescence microscopy. Human 293T cells were transiently transfected with expression vectors encoding hLhx3a-4xGFP. The fluorescence was detected using krypton-argon laser scanning confocal microscopy. Unlike 4xGFP control, which is restricted to the cytoplasm, hLhx3a-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 13E is an image depicting the localization of hLhx3b-4xGFP in cells. Human 293T cells were transiently transfected with expression vectors encoding hLhx3b-4xGFP. The cells were examined using phase contrast microscopy to visualize cell structures. Unlike control 4xGFP, which is restricted to the cytoplasm, hLhx3b-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 13F is an image depicting the localization of hLhx3b-4xGFP using fluorescence microscopy. Human 293T cells were transiently transfected with expression vectors encoding hLhx3b-4xGFP. The fluorescence was detected using krypton-argon

laser scanning confocal microscopy. Unlike 4xGFP control, which is restricted to the cytoplasm, hLhx3b-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 14A is a graph depicting a quantitative fluorescent assay for human Lhx3 gene expression. RNA was extracted from adult human pituitaries and cDNA was obtained using a 3' gene-specific primer as described elsewhere herein. PCR was performed using gene-specific primers and the PCR reactions were monitored in "real time" using an internal fluorescent hLhx3-specific probe (*i.e.*, a TaqMan probe as described elsewhere herein). The results are the means of four independent experiments ± SEM. The inset graph depicts a standard curve prepared using hLhx3 cDNA as input. In the inset graph, "Ct" denotes the PCR cycle number where the reporter dye fluorescent emission increases above baseline emission as described in Heid et al. (1996, Genome Res. 6:986-994).

Figure 14B is an image of a gel depicting a RT-PCR analysis of expression of transcripts of the hLhx3 gene encoding hLhx3a and hLhx3b isoforms. RT-PCR was used to amplify the approximately 1.2-kb coding regions of hLhx3a and hLhx3b using pituitary gland cDNA from two representative patients. Reaction products were separated by agarose gel electrophoresis and the gels were stained using ethidium bromide to visualize any RT-PCR amplicons. In the figure, the abbreviations used are as follows: M, molecular weight markers; Control, negative control.

Figure 14C is an image of a gel depicting the expression of Lhx3 isoforms in pituitary cell lines. RT-PCR was used to amplify specific regions of Lhx3a (139-bp product designated "a") and Lhx3b (165-bp product designated "b") using cDNA from the indicated cell lines. Amplification reaction products were separated by acrylamide gel electrophoresis and visualized by staining the gels using ethidium bromide. Negative control reactions (-) were performed in parallel in the absence of reverse transcriptase.

Figure 15A is a graph depicting differential activation of the alpha-glycoprotein subunit (αGSU) gene promoter by hLhx3 isoforms. Human 293 cells were transiently transfected with a mouse αGSU luciferase reporter gene (schematically diagramed along the top edge of the figure) and the indicated expression vector, *i.e.*, control vector without insert (control), vector comprising an insert encoding hLhx3a

(hLhx3a), vector comprising an insert encoding hLhx3a tagged with a myc-epitope (hLhx3a-myc), vector comprising an insert encoding hLhx3b (hLhx3b), and vector comprising an insert encoding hLhx3b tagged with a myc-epitope (hLhx3b-myc). Promoter activity was assayed by measurement of luciferase activity 48 hours post-transfection. Luciferase activities are the mean light units/10sec/μg total protein of triplicate assays ± SEM. A representative experiment of at least seven experiments is depicted.

Figure 15B is an image depicting a Western blot analysis using an anti-myc monoclonal antibody of cells transfected with control and myc epitope-tagged hLhx3 expression vectors demonstrating expression of hLhx3a and hLhx3b proteins in the cells. The migration positions of protein standards (in kilodaltons) are shown.

Figure 16A is a diagram depicting the induction of expression of a reporter gene construct containing the TSHβ promoter by hLhx3 isoforms and Pit-1 pituitary transcriptions factor. Human 293 cells were transiently transfected with a mouse TSHβ promoter reporter gene plasmid (as shown along the top edge of the figure) and hLhx3a, hLhx3b, and/or Pit-1 expression vectors. Luciferase activity was assayed 48 hours post-transfection. Values are the mean light units/10 sec/μg total protein of triplicate assays ± SEM. A representative experiment of at least four experiments is depicted.

Figure 16B is a diagram depicting the induction of expression of a reporter gene construct containing the TSHβ promoter by hLhx3 isoforms and thyrotrope embryonic factor (TEF) pituitary transcriptions factor. Human 293 cells were transiently transfected with a mouse TSHβ promoter reporter gene plasmid and hLhx3a, hLhx3b, and/or TEF expression vectors. Luciferase activity was assayed 48 hours post-transfection. Values are the mean light units/10 sec/μg total protein of triplicate assays ± SEM. A representative experiment of at least four experiments is depicted.

Figure 17 is a graph depicting the *trans*-activation of a luciferase reporter gene containing Lhx3 binding sites by hLhx3 isoforms. Human 293 cells were transiently transfected with a Lhx3 reporter gene containing three Lhx3 consensus sites and hLhx3a or hLhx3b expression vectors as depicted along the top edge of the figure. Reporter gene activity was measured 48 hours after transfection. The luciferase activities

disclosed are the mean light units/10 sec/μg total protein of triplicate assays ± SEM. A representative experiments of at least five experiments is depicted.

Figure 18A is an image of a gel depicting an analysis of the expression of recombinant hLhx3 proteins and analysis of Lhx3 isoforms binding of DNA target sequences. The image depicts a Coomassie brilliant blue stain of a SDS-polyacrylamide gel analysis of hLhx3a or hLhx3b fusion proteins containing a glutathione-S-transferase (GST) tag epitope. The migration of molecular weight standards is indicated in kilodaltons along the right-hand edge of the figure.

Figure 18B is an image of a gel depicting an electrophoretic mobility shift assay (EMSA) using Lhx3 consensus binding site (lanes 1-12) or αGSU gene -350 to -323 binding site (lanes 13 and 14) as oligonucleotide probes. Radiolabeled probes were incubated with the indicated proteins and competitor DNAs and the resulting complexes were separated from free probe (F) by electrophoresis. Lane 1, unprogrammed rabbit reticulocyte lysate as a negative control (lysate); lanes 2-5, reactions contained *in vitro* translated hLhx3 proteins including isoforms comprising a myc tag epitope. A nonspecific band is noted by an asterisk (*). Human Lhx3 protein/DNA complexes are indicated by an arrow. Lane 6 depicts the lack of binding using GST as a negative control; lanes 7-14 depict binding of the DNA probe by recombinant hLhx3 proteins expressed in *E. coli*. "Comp" denotes reactions containing approximately 1000-fold molar excess of unlabeled binding site as a competitor.

Figure 19 is a diagram depicting the nucleic acid sequence of human *Lhx3c* (SEQ ID NO:25).

Figure 20 is a diagram depicting the nucleic acid sequence of human *Lhx3d* (SEQ ID NO:26).

Figure 21 is a diagram depicting the nucleic acid sequence of human *Lhx3e* (SEQ ID NO:27).

Figure 22A is a diagram depicting the genomic organization of the human *LHX3* gene, more specifically, the structure of the *LHX3* gene. Exons are represented by boxes and labeled in Roman numerals. White boxes indicate untranslated regions; black boxes denote protein coding exons. Introns are labeled using Arabic numerals. The asterisk denotes the location of a conserved ATTTA motif. PCR primers: introns 1a and

1b: 5'-tgacctcggaggagcgcgtct-3' (SEQ ID NO:46) and 5'-tcgtccttgcagtaaacgct-3' (SEQ ID NO:47); intron 2: 5'-agcgtttactgcaaggacga-3' (SEQ ID NO:48) and 5'-cgcacttggtcccgaagcgc-3' (SEQ ID NO:49); introns 3 and 4: 5'-gcgcttcgggaccaagtgcg-3' (SEQ ID NO:50) and 5'-cggggaaggagacctcagcgt-3' (SEQ ID NO:51); Intron 5: 5'-ggacaaggacagcgttcag-3' (SEQ ID NO:52) and 5'-ctcccgtagaggccattg-3' (SEQ ID NO:53).

Figure 22B is a diagram depicting the location of CpG dinucleotide sequences (vertical lines) within the *LHX3* locus.

Figure 22C is a diagram depicting the structure of LHX3 protein isoforms and correlation of protein domains with the gene exon structure.

Figure 23A is an image depicting the chromosomal localization of the human *LHX3* gene by fluorescence *in situ* hybridization (FISH). The image depicts a metaphase chromosome spread demonstrating the presence of the *LHX3* gene (red, large arrow) on both Chr 9s identified by the green centromeric Chr 9 probe (CEP 9; VYSIS Inc.) indicated by the small arrow at the terminal region of band 9q34.3.

Figure 23B is an image of an ideogram of Chromosome 9 demonstrating the location of *LHX3*.

Figure 23C is an image depicting the co-localization of *LHX3* gene probe (red, large arrow) with the green chromosome subtelomeric probe (TelVysion 9q; VYSIS Inc.). Note the yellow color resulting from the close proximity of the red and green labeled probes. The centromere is marked using a green fluorescent probe (CEP 9; VYSIS Inc.) indicated by the small arrow.

Figure 24 is an image depicting the amino acid sequence of human Lhx3a (SEQ ID NO:10). The amino acids of LIM Domain 1 (amino acids 31-81) and LIM Domain 2 (amino acids 90-144) are underlined. Further, the amino acids of homeodomain (amino acids 156-220) are underlined. The protein is about 397 amino acids in length.

Figure 25 is an image depicting the amino acid sequence of human Lhx3b (SEQ ID NO:12). The amino acids of LIM Domain 1 (amino acids 36-86) and LIM Domain 2 (amino acids 95-149) are underlined. Further, the amino acids of homeodomain (amino acids 161-225) are underlined. The protein is about 402 amino acids in length.

Figure 26A-D is an image depicting the nucleic acid sequence of the genomic DNA encoding human Lhx3 (SEQ ID NO:22). Nucleotides representing introns are capitalized.

Figure 27 is an image depicting the nucleic acid sequence of porcine *Lhx3a* (SEQ ID NO:13).

Figure 28 is an image depicting the amino acid sequence of porcine Lhx3a (SEQ ID NO:14).

Figure 29 is an image depicting the nucleic acid sequence of porcine *Lhx3b* (SEQ ID NO:15).

Figure 30 is an image depicting the amino acid sequence of porcine Lhx3b (SEQ ID NO:16).

BRIEF DESCRIPTION OF THE DRAWINGS

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~~The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.~~

In the drawings:

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Figure 12A is an image of a Southern blot depicting the fact that hLhx3 is encoded by single gene. Human genomic DNA was digested with EcoRI and blotted to a nylon membrane. The blot was probed with a hLhx3 cDNA probe at high stringency. The migration position of molecular weight standards (kb) is given.

Figure 12B is an image of a Northern blot depicting analysis of hLhx3 gene expression. Human kidney (lane 2) or pituitary (Pit.; lane 1) total (20 μg) or poly A^+ RNA (1 μg; lanes 3 and 4) was separated on a denaturing gel, transferred to a nylon

membrane, and probed with the indicated radiolabeled cDNA probes (*e.g.*, Lhx3 and Pit-1). The migration positions of ribosomal RNAs (*i.e.*, 28S and 18S) are indicated. The arrowhead indicates hLhx3 RNA.

Figure 12C is an image of SDS-PAGE analysis of human Lhx3a and hLhx3b proteins. [^{35}S]-methionine radiolabeled hLhx3a and hLhx3b isoforms (lanes 1 and 2) and myc epitope-tagged derivatives (a-myc, b-myc; lanes 4 and 5) were generated by *in vitro* transcription/translation. The proteins were separated by SDS electrophoresis, and dried gels were visualized using fluorography. The migration positions of protein standards (in kilodaltons) are shown. Lane 3 is control.

Figure 13A is an image depicting the localization of control green fluorescent protein (GFP) in cells. Human 293T cells were transiently transfected with expression vectors encoding multimerized green fluorescent protein (GFP) as a control (4xGFP). The cells were examined using phase contrast microscopy. Bar = 10 μm.

Figure 13B is an image depicting the localization of control GFP protein using fluorescence microscopy. Human 293T cells were transiently transfected with expression vectors encoding multimerized green fluorescent protein (GFP) as a control (4xGFP). The fluorescence was visualized using krypton-argon laser scanning confocal microscopy. 4xGFP control is restricted to the cytoplasm. Bar = 10 μm.

Figure 13C is an image depicting the localization of hLhx3a-GFP in cells. Human 293T cells were transiently transfected with expression vectors encoding hLhx3a-4xGFP. The cells were examined using phase contrast microscopy to visualize cell structures. Unlike control 4xGFP, which is restricted to the cytoplasm, hLhx3a-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 13D is an image depicting the localization of hLhx3a-GFP using fluorescence microscopy. Human 293T cells were transiently transfected with expression vectors encoding hLhx3a-4xGFP. The fluorescence was detected using krypton-argon laser scanning confocal microscopy. Unlike 4xGFP control, which is restricted to the cytoplasm, hLhx3a-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 13E is an image depicting the localization of hLhx3b-4xGFP in cells. Human 293T cells were transiently transfected with expression vectors encoding hLhx3b-4xGFP. The cells were examined using phase contrast microscopy to visualize

cell structures. Unlike control 4xGFP, which is restricted to the cytoplasm, hLhx3b-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 13F is an image depicting the localization of hLhx3b-4xGFP using fluorescence microscopy. Human 293T cells were transiently transfected with expression vectors encoding hLhx3b-4xGFP. The fluorescence was detected using krypton-argon laser scanning confocal microscopy. Unlike 4xGFP control, which is restricted to the cytoplasm, hLhx3b-4xGFP is detected in the nuclei of transfected cells. Bar = 10 μm.

Figure 14A is a graph depicting a quantitative fluorescent assay for human Lhx3 gene expression. RNA was extracted from adult human pituitaries and cDNA was obtained using a 3' gene-specific primer as described elsewhere herein. PCR was performed using gene-specific primers and the PCR reactions were monitored in "real time" using an internal fluorescent hLhx3-specific probe (*i.e.*, a TaqMan probe as described elsewhere herein). The results are the means of four independent experiments ± SEM. The inset graph depicts a standard curve prepared using hLhx3 cDNA as input. In the inset graph, "Ct" denotes the PCR cycle number where the reporter dye fluorescent emission increases above baseline emission as described in Heid et al. (1996, Genome Res. 6:986-994).

Figure 14B is an image of a gel depicting a RT-PCR analysis of expression of transcripts of the hLhx3 gene encoding hLhx3a and hLhx3b isoforms. RT-PCR was used to amplify the approximately 1.2-kb coding regions of hLhx3a and hLhx3b using pituitary gland cDNA from two representative patients. Reaction products were separated by agarose gel electrophoresis and the gels were stained using ethidium bromide to visualize any RT-PCR amplicons. In the figure, the abbreviations used are as follows: M, molecular weight markers; Control, negative control.

Figure 14C is an image of a gel depicting the expression of Lhx3 isoforms in pituitary cell lines. RT-PCR was used to amplify specific regions of Lhx3a (139-bp product designated "a") and Lhx3b (165-bp product designated "b") using cDNA from the indicated cell lines. Amplification reaction products were separated by acrylamide gel electrophoresis and visualized by staining the gels using ethidium bromide. Negative control reactions (-) were performed in parallel in the absence of reverse transcriptase.

Figure 15A is a graph depicting differential activation of the alpha-glycoprotein subunit (αGSU) gene promoter by hLhx3 isoforms. Human 293 cells were transiently transfected with a mouse αGSU luciferase reporter gene (schematically diagramed along the top edge of the figure) and the indicated expression vector, *i.e.*, control vector without insert (control), vector comprising an insert encoding hLhx3a (hLhx3a), vector comprising an insert encoding hLhx3a tagged with a myc-epitope (hLhx3a-myc), vector comprising an insert encoding hLhx3b (hLhx3b), and vector comprising an insert encoding hLhx3b tagged with a myc-epitope (hLhx3b-myc). Promoter activity was assayed by measurement of luciferase activity 48 hours post-transfection. Luciferase activities are the mean light units/10sec/μg total protein of triplicate assays ± SEM. A representative experiment of at least seven experiments is depicted.

Figure 15B is an image depicting a Western blot analysis using an anti-myc monoclonal antibody of cells transfected with control and myc epitope-tagged hLhx3 expression vectors demonstrating expression of hLhx3a and hLhx3b proteins in the cells. The migration positions of protein standards (in kilodaltons) are shown.

Figure 16A is a diagram depicting the induction of expression of a reporter gene construct containing the TSHβ promoter by hLhx3 isoforms and Pit-1 pituitary transcriptions factor. Human 293 cells were transiently transfected with a mouse TSHβ promoter reporter gene plasmid (as shown along the top edge of the figure) and hLhx3a, hLhx3b, and/or Pit-1 expression vectors. Luciferase activity was assayed 48 hours post-transfection. Values are the mean light units/10 sec/μg total protein of triplicate assays ± SEM. A representative experiment of at least four experiments is depicted.

Figure 16B is a diagram depicting the induction of expression of a reporter gene construct containing the TSHβ promoter by hLhx3 isoforms and thyrotrope embryonic factor (TEF) pituitary transcriptions factor. Human 293 cells were transiently transfected with a mouse TSHβ promoter reporter gene plasmid and hLhx3a, hLhx3b, and/or TEF expression vectors. Luciferase activity was assayed 48 hours post-transfection. Values are the mean light units/10 sec/μg total protein of triplicate assays ± SEM. A representative experiment of at least four experiments is depicted.

Figure 17 is a graph depicting the *trans*-activation of a luciferase reporter gene containing Lhx3 binding sites by hLhx3 isoforms. Human 293 cells were transiently transfected with a Lhx3 reporter gene containing three Lhx3 consensus sites and hLhx3a or hLhx3b expression vectors as depicted along the top edge of the figure. Reporter gene activity was measured 48 hours after transfection. The luciferase activities disclosed are the mean light units/10 sec/μg total protein of triplicate assays ± SEM. A representative experiments of at least five experiments is depicted.

Figure 18A is an image of a gel depicting an analysis of the expression of recombinant hLhx3 proteins and analysis of Lhx3 isoforms binding of DNA target sequences. The image depicts a Coomassie brilliant blue stain of a SDS-polyacrylamide gel analysis of hLhx3a or hLhx3b fusion proteins containing a glutathione-S-transferase (GST) tag epitope. The migration of molecular weight standards is indicated in kilodaltons along the right-hand edge of the figure.

Figure 18B is an image of a gel depicting an electrophoretic mobility shift assay (EMSA) using Lhx3 consensus binding site (lanes 1-12) or αGSU gene -350 to -323 binding site (lanes 13 and 14) as oligonucleotide probes. Radiolabeled probes were incubated with the indicated proteins and competitor DNAs and the resulting complexes were separated from free probe (F) by electrophoresis. Lane 1, unprogrammed rabbit reticulocyte lysate as a negative control (lysate); lanes 2-5, reactions contained *in vitro* translated hLhx3 proteins including isoforms comprising a myc tag epitope. A nonspecific band is noted by an asterisk (*). Human Lhx3 protein/DNA complexes are indicated by an arrow. Lane 6 depicts the lack of binding using GST as a negative control; lanes 7-14 depict binding of the DNA probe by recombinant hLhx3 proteins expressed in *E. coli*. "Comp" denotes reactions containing approximately 1000-fold molar excess of unlabeled binding site as a competitor.

Figure 19 is a diagram depicting the nucleic acid sequence of human *Lhx3c* (SEQ ID NO:25).

Figure 20 is a diagram depicting the nucleic acid sequence of human *Lhx3d* (SEQ ID NO:26).

Figure 21 is a diagram depicting the nucleic acid sequence of human *Lhx3e* (SEQ ID NO:27).

Figure 22A is a diagram depicting the genomic organization of the human *LHX3* gene, more specifically, the structure of the *LHX3* gene. Exons are represented by boxes and labeled in Roman numerals. White boxes indicate untranslated regions; black boxes denote protein coding exons. Introns are labeled using Arabic numerals. The asterisk denotes the location of a conserved ATTTA motif. PCR primers: introns 1a and 1b: 5'-tgacctcggaggagcgcgtct-3' (SEQ ID NO:46) and 5'-tcgtccttgcagtaaacgct-3' (SEQ ID NO:47); intron 2: 5'-agcgtttactgcaaggacga-3' (SEQ ID NO:48) and 5'-cgcacttggtcccgaagcgc-3' (SEQ ID NO:49); introns 3 and 4: 5'-gcgcttcgggaccaagtgcg-3' (SEQ ID NO:50) and 5'-cggggaaggagacctcagcgt-3' (SEQ ID NO:51); Intron 5: 5'-ggacaaggacagcgttcag-3' (SEQ ID NO:52) and 5'-ctcccgtagaggccattg-3' (SEQ ID NO:53).

Figure 22B is a diagram depicting the location of CpG dinucleotide sequences (vertical lines) within the *LHX3* locus.

Figure 22C is a diagram depicting the structure of LHX3 protein isoforms and correlation of protein domains with the gene exon structure.

Figure 23A is an image depicting the chromosomal localization of the human *LHX3* gene by fluorescence *in situ* hybridization (FISH). The image depicts a metaphase chromosome spread demonstrating the presence of the *LHX3* gene (red, large arrow) on both Chr 9s identified by the green centromeric Chr 9 probe (CEP 9; VYSIS Inc.) indicated by the small arrow at the terminal region of band 9q34.3.

Figure 23B is an image of an ideogram of Chromosome 9 demonstrating the location of *LHX3*.

Figure 23C is an image depicting the co-localization of *LHX3* gene probe (red, large arrow) with the green chromosome subtelomeric probe (TelVysion 9q; VYSIS Inc.). Note the yellow color resulting from the close proximity of the red and green labeled probes. The centromere is marked using a green fluorescent probe (CEP 9; VYSIS Inc.) indicated by the small arrow.

Figure 24 is an image depicting the amino acid sequence of human Lhx3a (SEQ ID NO:10). The amino acids of LIM Domain 1 (amino acids 31-81) and LIM Domain 2 (amino acids 90-144) are underlined. Further, the amino acids of homeodomain (amino acids 156-220) are underlined. The protein is about 397 amino acids in length.

Figure 25 is an image depicting the amino acid sequence of human Lhx3b (SEQ ID NO:12). The amino acids of LIM Domain 1 (amino acids 36-86) and LIM Domain 2 (amino acids 95-149) are underlined. Further, the amino acids of homeodomain (amino acids 161-225) are underlined. The protein is about 402 amino acids in length.

Figure 26A-D is an image depicting the nucleic acid sequence of the genomic DNA encoding human Lhx3 (SEQ ID NO:22). Nucleotides representing introns are capitalized.

Figure 27 is an image depicting the nucleic acid sequence of porcine *Lhx3a* (SEQ ID NO:13).

Figure 28 is an image depicting the amino acid sequence of porcine Lhx3a (SEQ ID NO:14).

Figure 29 is an image depicting the nucleic acid sequence of porcine *Lhx3b* (SEQ ID NO:15).

Figure 30 is an image depicting the amino acid sequence of porcine Lhx3b (SEQ ID NO:16).